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   865 SW 78th Avenue
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   Plantation
   Florida 33324, USA

   CO80749 5002
   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — USA

4. Title of the invention — "Protein Production in Transgenic Avians"

5. Name of your agent *(if you have one)*

   Murgitroyd & Company

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   Scotland House
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"Protein Production in Transgenic Avians"

The present invention relates to the generation of transgenic avians and the production of recombinant proteins. More particularly, the invention relates to the enhanced transduction of avian cells by exogenous genetic material so that the genetic material is incorporated into an avian genome in such a way that the modification becomes integrated into the germline and results in expression of the encoded protein within the avian egg.

The ability to manufacture large amounts of pharmaceutical grade proteins is becoming increasingly important in the biotechnology and pharmaceutical arenas. Recent successes of such products in the marketplace, especially those of monoclonal antibodies, have put an enormous strain on already stretched global manufacturing facilities. This heightened demand for manufacturing capacity, the consequential high premiums on capacity and the long wait for

production space, plus the cost of and issues involved in producing proteins in cell lines, has prompted companies to look beyond traditional modes of production (Andersson & Myhanan, 2001). Traditional methods for manufacture of recombinant proteins include production in bacterial or mammalian cells. One of the alternative manufacturing strategies is the use of transgenic animals and plants for production of proteins.

It was by genetic engineering that the first genetically modified (transgenic) animal was produced, by transferring the gene for the protein of interest into the target animal. Current transgenic technology can be traced back to a series of pivotal experiments conducted between 1968 and 1981 including: the generation of chimeric mice by blastocyst injection of embryonic stem cells (Gardner *et al* 1968), the delivery of foreign DNA to rabbit oocytes by spermatozoa (Brackett *et al* 1971), the production of transgenic mice made by injecting viral DNA into pre-implantation blastocysts (Jaenisch *et al* 1974) and germline transmission of transgenes in mouse by pronuclear injection (Gordon and Ruddle, 1981). For the early part of transgenics' history, the focus was upon improving the genetic makeup of the animal and thus the yield of wool, meat or eggs (Curtis & Barnes, 1989; Etches & Gibbins, 1993). However in recent years there has been interest in utilising transgenic systems for medical applications such as organ transplantation,

models for human disease or for the production of proteins destined for human use.

A number of protein based biopharmaceuticals have been produced in the milk of transgenic mice, rabbits, pigs, sheep, goats and cows at reasonable levels, but such systems tend to have long generation times - some of the larger mammals can take years to develop from the founder transgenic to a stage at which they can produce milk. Additional difficulties relate to the biochemical complexity of milk and the evolutionary conservation between humans and mammals, which can result in adverse reactions to the pharmaceutical in the mammals which are producing it (Harvey et al., 2002).

There is increasing interest in the use of chicken eggs as a potential manufacturing vehicle for pharmaceutically important proteins, especially recombinant human antibodies. Huge amounts of therapeutic antibodies are required by the medical community each year, amounts which can be kilogram or metric tons per year, so a manufacturing methodology which could address this shortage would be a great advantage. Once optimised, a manufacturing method based on chicken eggs has several advantages as compared to mammalian cell culture or use of transgenic mammalian systems. Firstly, chickens have a short generation time (24 weeks), which would allow transgenic flocks to be established rapidly. Table 1 shows a comparison between the different types of transgenic systems. Secondly, the capital outlays for a transgenic

animal production facility are far lower than that for cell culture. Extra processing equipment is minimal in comparison to that required for cell culture (BioPharm, 2001). As a consequence of these lower capital outlays, the production cost per unit of therapeutic will be lower than that produced by cell culture. In addition, transgenic systems provide significantly greater flexibility regarding purification batch size and frequency and this flexibility may lead to further reduction of capital and operating costs in purification through batch size optimisation. The third advantage of increased speed to market should become apparent when the technology has been developed to a commercially viable degree. Transgenic mammals are capable of producing several grams of protein product per litre of milk, making large-scale production commercially viable (Weck, 1999). Mammals do not have a significant advantage in terms of the time take to scale up production, since gestation periods for cows and goats are 9 months and 5 months respectively (Dove, 2000) and it can take up to five years to produce a commercially viable herd. However, once the herd is established, the yield of product from milk will be high.

Animal	Gestation	Maturity/ Generation time	Offspring Produced	Time to Production Herd/Flock	Protein (per litre/ egg per day)	Founder animal development cost
Cow	9 months	2 years	1 per year	5+ years	15g	$5-10M
Goat	5 months	8 months	2-4 per year	3-5 years	8g	$3M
Sheep	5 months	8 months	2 per year	3-5 years	8g	$2M
Pigs	4 months	8 months	10	?	4.1g	?
Rabbits	1 month	5 months	8	?	0.05g	?
Chicken	21 days	6 months	21 per month	18 months	0.3g	$0.25M

Table 1. A comparison between the various transgenic animal production system (Dove, 2000).

The short generation time for birds also allows for rapid scale-up. The incubation period of a chicken is only 21 days and it reaches maturity within six months of hatch. Indeed, once the founder animals of the flock have been established, a flock can be established within 18 months (Dove, 2000). The process of scaling up the production capability should be simpler and far faster than a herd of sheep, goats or cows.

A further advantage rests in the fact that eggs are naturally sterile vessels. One of the inherent problems with cell culture methods of production is the risk of microbial contamination, since the nutrient rich media used tends to encourage microbial growth. Transgenic production offers a lower risk alternative, since the production of the protein will occur within the animal itself, whose own body will combat most infections. Chicken eggs provide an even lower risk alternative: the eggs are

sealed within the shell and membrane and thus separated from the environment. The evolutionary distance between humans and birds means that few diseases are common to both.

Still a further potential advantage lies in the post-translational modification of chicken proteins. The issue of how well a production process can reproduce the natural sugar profile on the proteins which are produced, is now recognised as a crucial element of the success of a production technology (Morrow, 2001; Raju *et al.*, 2000). The main cell types used in cell culture processes are either hamster or mouse-derived, so do not produce the same sugar pattern on proteins as human cells (Scrip, June 8th 2001). Mammalian and particularly plant transgenic systems produce different types of post-translational modifications on expressed proteins. The sugar profile is crucially important to the manner in which the human immune system reacts to the protein. Raju *et al.*, (2000) found that glycosylated chicken proteins have a sugar profile that is more similar to that of glycosylated human proteins than non-human mammalian proteins, which should be a significant advantage in developing a therapeutic product.

It can therefore be seen that the avian egg, particularly from the chicken, offers several major advantages over cell culture as a means of production and the other transgenic production systems based upon mammals or plants.

Direct application of the methods used in the production of transgenic mammals to the genetic manipulation of birds has not been possible because of specific features of the reproductive system of the laying hen. Following either natural or artificial insemination, hens will lay fertile eggs for approximately 10 days. They ovulate once per day, and fertilisation occurs almost immediately, while the ovum is at the top of the oviduct. The egg spends the next 20-24 hours in the oviduct, where the albumen (egg white) is laid down around the yolk, plumping fluid is added to the albumen and finally the shell membranes and the shell itself are laid down. During this time, cell division is rapid, such that by the time the egg is laid, the embryo comprises a blastoderm, a disc of approximately 60,000 relatively undifferentiated cells, lying on the yolk.

The complexities of egg formation make the earliest stages of chick-embryo development relatively inaccessible. Methods employed to access earlier stage embryos usually involve sacrificing the donor hen to obtain the embryo or direct injection into the oviduct. Methods for the production of transgenic mammals have focused almost exclusively on the microinjection of a fertilised egg, whereby a pronucleus is microinjected in vitro with DNA and the manipulated eggs are transferred to a surrogate mother for development to term, this method is not feasible in hens. Four general methods for the creation of transgenic avians have been developed.

A method for the production of transgenic chickens using DNA microinjection into the cytoplasm of the germinal disk was developed. The chick zygotes are removed from the oviduct of laying hens before the first cleavage division, transferred to surrogate shells, manipulated and cultured through to hatch (Perry 1998: Roslin US5011780 and EP0295964). Love *et al* 1994 analysed the embryos that survived for at least 12 days in culture and showed that approximately half of the embryos contained plasmid DNA, with 6% at a level equivalent to one copy per cell. Seven chicks, 5.5% of the total number of ova injected, survived to sexual maturity. One of these, a cockerel identified as a potential mosaic transgenic bird, transmitted the transgene to 3.4% of his offspring. These birds have been bred to show stable transmission of the transgene. As in transgenic mice generated by pro-nuclear injection, integration of the plasmid DNA is apparently a random event. However, direct DNA microinjection into eggs results in low efficiencies of transgene integration (Sang & Perry, 1989). It has been estimated that only 1% of microinjected ova give rise to transgenic embryos and of these 10% survive to hatch. The efficiency of this method could be improved by increasing the survival rate of the cultured embryos and the frequency of chromosomal integration of the injected DNA.

A second method involves the transfection of primordial germ cells *in vitro* and transplantation into a suitably prepared recipient. Successful

transfer of primordial germ cells has been achieved, resulting in production of viable gametes from the transferred germ cells. Transgenic offspring, as a result of gene transfer to the primordial germ cells before transfer, have not yet been described.

The third method involves the use of gene transfer vectors derived from oncogenic retroviruses. The early vectors were replication competent (Salter et al.) but replication defective vectors have been developed (see, eg. US Patent 5,162,215 and WO 97/47739). These systems use either the reticuloendotheliosis virus type A (REV-A) or avian leukosis virus (ALV). The efficiency of these vectors, in terms of production of founder transgenic birds, is low and inheritance of the vector from these founders is also inefficient (Harvey et al., 2002). These vectors may also be affected by silencing of expression of the transgenes they carry as reports suggest that protein expression levels are low (Harvey et al., 2002).

The fourth method involves the culture of chick embryo cells *in vitro* followed by production of chimeric birds by introduction of these cultured cells into recipient embryos (Pain et al., 1996). The embryo cells may be genetically modified *in vitro* before chimera production, resulting in chimeric transgenic birds. No reports of germline transmission from genetically modified cells are available.

Although much work has been carried out on retroviral vectors derived from viruses such as ALV and REV as mentioned previously, the limitations of such vectors have prevented more widespread application. Much of the research and development of viral vectors was based on their use in gene therapy applications and so resulted in the demonstration that vectors based on lentiviruses were able to infect nondividing cells, a clear advantage in clinical gene therapy applications. Lentiviruses are a subgroup of the retroviruses which include a variety of primate viruses eg. human immunodeficiency viruses HIV-1 and 2 and simian immunodeficiency viruses (SIV) and non-primate viruses (eg. maedi-visna virus (MVV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), caprine arthrithis encephalitis virus (CAEV) and bovine immunodeficiency virus (BIV). These viruses are of particular interest in development of gene therapy treatments, since not only do the lentiviruses possess the general retroviral characteristics of irreversible integration into the host cell DNA, but as mentioned previously, also have the ability to infect non-proliferating cells. The dependence of other types of retroviruses on the cell proliferation status has somewhat limited their use as gene transfer vehicles. The biology of lentiviral infection can be reviewed in Coffin *et al.*, 1997 and Sanjay *et al.*, 1996.

An important consideration in the design of a viral vector is the ability to be able to stably integrate into the genome of cells. Previous work has shown that oncoretroviral vectors used as gene transfer vehicles have had somewhat limited success due to the gene silencing effects during development. Jahner et al., (1982) showed that use of the vector based on the Moloney murine leukemia virus (MoMLV) for example, is unsuitable for production of transgenic animals due to silencing of the virus during the developmental phase, leading to very low expression of the transgene. It is therefore essential that any viral vector used for production of transgenic birds does not exhibit gene silencing. The work of Pfeifer *et al.*, 2001 and Lois *et al.*, 2002 on mice has shown that a lentiviral vector based on HIV-1 is not silenced during development.

The bulk of the developmental work on lentiviral vectors has been focused upon HIV-1 systems, largely due to the fact that HIV, by virtue of its pathogenicity in humans, is the most fully characterised of the lentiviruses. Such vectors tend to be engineered as to be replication incompetent, through removal of the regulatory and accessory genes, which render them unable to replicate. The most advanced of these vectors have been minimised to such a degree that almost all of the regulatory genes and all of the accessory genes have been removed. However, due to the pathogenicity of HIV in humans, there has been a move away from HIV as a base. Clearly from the

point of view of developing a production technology which will be used to manufacture therapeutics destined for human use, it would be preferable to develop vectors based on lentiviruses which are non-pathogenic to humans. The lentiviral group have many similar characteristics, such as a similar genome organisation, a similar replication cycle and the ability to infect mature macrophages (Clements & Payne, 1994). One such lentivirus is Equine Infectious Anemia Virus (EIAV) Compared with the other viruses of the lentiviral group, EIAV has a relatively simple genome: in addition to the retroviral *gag*, *pol* and *env* genes, the genome only consists of three regulatory/accessory genes (*tat*, *rev* and *S2*). The development of a safe and efficient lentiviral vector system will be dependent on the design of the vector itself. It is important to minimise the viral components of the vector, whilst still retaining its transducing vector function. A vector system derived from EIAV has been shown to transduce dividing and non-dividing cells with similar efficiencies to HIV-based vectors (Mitrophanous et al., 1999). Oncoretroviral and lentiviral vectors systems may be modified to broaden the range of tranducible cell types and species. This is achieved by substituting the envelope glycoprotein of the virus with other virus envelope proteins. These include the use of the amphotropic MLV envelope glycoprotein (Page et al., 1990) or the vesicular stomatitis virus G-protein (VSV-G) (Yee et al., 1994). The use of VSV-G pseudotyping also results in greater stability of

the virus particles and enables production of virus at higher titres.

It is an aim of the present invention to provide an efficient method for transferring a transgene construct to avian embryonic cells so as to create a transgenic bird which expresses the gene in its tissues, especially, but not exclusively, in the cells lining the oviduct so that the translated protein becomes incorporated into the produced eggs.

It is also an aim of the present invention to provide a vehicle and a method for transferring a gene to avian embryonic cells so as to create a transgenic bird which has stably incorporated the transgene into a proportion or all of its germ cells, resulting in transmission of the transgene to a proportion of the offspring of the transgenic bird. This germ line transmission will result in a proportion of the offspring of the founder bird exhibiting the altered genotype.

According to the present invention there is provided a method for the production of transgenic avians, the method comprising the step of using a lentivirus vector system to deliver exogenous genetic material to avian embryonic cells.

The lentivirus vector system includes a lentivirus transgene construct in a form which is capable of being delivered to and integrated with the genome of avian embryonic cells.

In one embodiment the lentivirus construct is injected into the subgerminal cavity of the contents of an opened egg which is then allowed to develop using the Perry Culture system of surrogate shells.

In another embodiment the construct is injected directly into the sub-blastodermal cavity of an egg.

Typically the genetic material encodes a protein.

The invention thus provides a transgenic avian.

Preferably the transgenic avian produced by the method of the invention has the genetic material incorporated into at least a proportion of germ cells such that the genetic material will be transmitted to at least a proportion of the offspring of the transgenic avian.

The invention thus provides further transgenic avians.

According to the present invention there is also provided a method for production of an heterologous protein in avians, the method comprising the step of delivering genetic material encoding the protein within a lentivirus vector construct to avian embryonic cells so as to create a transgenic avaian which expresses the genetic material in its tissues. Preferably the transgenic avian expresses the gene in the oviduct so that the translated protein becomes incorporated into eggs.

The protein can then be isolated from eggs by known methods.

The invention provides the use of a lentivirus construct for the production of transgenic avians.

The invention also provides the use of a lentivirus vector construct for the production of proteins in transgenic avians.

Preferably the lentivirus vector construct is used for the expression of heterologous proteins in specific tissues, preferably egg white or yolk.

The lentivirus as used in this application may be any lentiviral vector but is preferably chosen from the group consisting of EIAV, HIV, SIV, BIV and FIV.

A particularly preferred vector is EIAV.

Any commercially available lentivirus vector may be suitable to be used as a basis for a construct to deliver exogeneous genetic material.

Preferably the construct includes suitable enhancer promoter elements for subsequent production of protein.

Preferably the vector construct particles are packaged using a commercially available packaging system to produce vector with an envelope, typically a VSV-G envelope.

Typically the vector may be based on EIAV available from ATCC under accession number VR-778 or other commercially available vectors.

Commercial lentivirus-based vectors for use in the methods of the invention are capable of infecting a wide range of species without producing any live virus and do not cause cellular or tissue toxicity.

The methods of the present invention can be used to generate any transgenic avian, including but not limited to chickens, turkeys, ducks, quail, geese, ostriches, pheasants, peafowl, guinea fowl, pigeons, swans and penguins.

These lentivirus-based vector systems also have a large transgene capacity which are capable of carrying larger protein encoding constructs such as antibody encoding constructs.

A preferred lentiviral vector system is the LentiVector® system of Oxford BioMedica.

The invention is exemplified with reference to the following non-limiting examples.

Freshly laid, fertile hen's eggs were obtained which contain developing chick embryos at developmental stages X-XIII (Eyal-Giladi and Kochav, 1976). An egg was opened, the contents transferred to a dish and 2-3 microlitres of a suspension of lentiviral vector virus particles was injected into the subgerminal

cavity, below the developing embryo but above the yellow yolk. The vector used was derived from Equine Infectious Anaemia Virus (EIAV) and carried a reporter gene, β-galactosidase (lacZ), under the control of the CMV (cytomegalovirus) enhancer/promoter. The packaging system used to generate the vector viral particles resulted in production of the vector with a VSV-G envelope. The estimated concentration of viral transducing particles was between 5×10^7 and 1×10^9 per ml. The embryos were allowed to develop by culturing them using the second and third phases of the Perry culture system (Perry, 1988). 12 embryos were removed and analysed for expression of lacZ after 2 days of incubation and 12 embryos after 3 days of incubation. The embryos and surrounding membranes were dissected free of yolk, fixed and stained to detect expression of the lacZ reporter gene. All embryos showed expression of lacZ in some cells of the embryo and surrounding membranes. The expression was highest in the developing extraembryonic membrane close to the embryo and was limited to a small number of cells in the embryos analysed. These results indicated that all the embryos had been successfully transduced by the injected lentiviral vector.

In a further experiment 40 laid eggs were injected each with 2-3 microlitres of a suspension of the EIAV vector at a titre of 5×10^8 per ml., into the sub-blastodermal cavity. 13 chicks hatched (33%) and were screened to identify transgenic offspring

carrying the lentiviral vector sequence. Samples of the remaining extraembryonic membrane were recovered from individual chicks after hatch, genomic DNA extracted and the DNA analysed by PCR using primers specific to the lentiviral vector sequence. The screen identified 11 chicks as transgenic (85%). The vector sequence was detected in the extraembryonic membrane at a copy number of between 0.4% and 31%, indicating that the chicks were mosaic for integration of the vector. This result was predicted as the embryos were injected with the vector at a stage at which they consisted of at least 60,000 cells. It is unlikely that all the cells in the embryo would be transduced by the viral vector, resulting in chicks that were chimeric for integration of the vector. The 11 chicks were raised to sexual maturity and 7 found to be males. Semen samples were obtained from the cockerels when they reached 16-20 weeks of age. DNA from these samples was screened by PCR and the seven cockerels found to have lentiviral vector sequence in the semen at levels estimated as between 0.1% and 80%. The majority of the samples contained vector sequence at a level above 10%. This suggested that at least 10% of the offspring of these cockerels will be transgenic. Semen was collected from one cockerel, code no. LEN5-20, that had been estimated to have a copy number of the viral vector in DNA from a blood sample as 6%. The copy number estimated from the semen sample was 80%. The semen was used to inseminate stock hens, and the fertile eggs collected and incubated. 9 embryos were recovered

after 3 days of incubation, screened by PCR to identify transgenic embryos and stained for expression of the lacZ reporter gene. 3 of the 9 embryos were transgenic and all 3 expressed lacZ but at a very low level in a small number of cells. 12 embryos were recovered after 10 days of incubation and screened as above. 6 embryos were identified as transgenic and lacZ expression detected in 4. The expression was high in several tissues in one embryo and lower in the other 3. These results indicate that 43% of the offspring of cockerel LEN5-20 were transgenic. The expression of the reporter construct carried by the lentiviral vector varied between individual transgenic chicks. It is likely that the individual chicks had copies of the vector genome integrated at different chromosomal sites, which may affect the expression of the transgene. It is also possible that some chicks carried more than one copy of the transgene.

The results outlined here demonstrate that a specific EIAV-derived lentiviral vector, pseudotyped with the VSV envelope protein, can transduce the germ cells of chick embryos with very high efficiency. The resulting birds then transmit the integrated vector to a high proportion of their offspring. The transgene carried by the vector may be expressed to give a functional protein at relatively high levels. The transgene carried by the vector may be designed to express foreign proteins at high levels in specific tissues.

The lentiviral vector may be introduced into the chick at different developmental stages, using modifications of the method described in the example above.

The viral suspension may be injected above the blastoderm embryo in a new laid egg .
The viral suspension may be injected into the newly fertilised egg or the early cleavage stages, up to stageX (Eyal-Giladi and Kochav), by utilizing the culture method of Perry (1988) or recovering eggs from the oviduct and then returning them to a recipient hen by ovum transfer.

The viral suspension may be injected above or below the blastoderm embryo in a freshly laid egg which has been accessed by cutting a window in the shell. The window may be resealed and the egg incubated to hatch (Bosselman et. al., 1989).

The viral suspension may be injected into the testes of cockerels and semen screened to detect transduction of the spermatogonia and consequent development of transgenic sperm.

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